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The simian virus 40 minimal origin and the 72-base-pair repeat are required simultaneously for efficient induction of late gene expression with large tumor antigen

(late promoter region/deletion and point mutagenesis/chloramphenicol acetyltransferase gene vector/transient expression assay/induction signals)

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ABSTRACT **We have studied the temporal regulation of simian virus 40 (SV40) late gene expression by construction and transient expression analysis of plasmids containing the transposon Tn*9* chloramphenicol acetyltransferase gene placed downstream from the late control region. The SV40 origin region in the early (but not the late) orientation promotes chloramphenicol acetyltransferase gene expression efficiently in monkey cells lacking large tumor (T) antigen. In monkey cells producing T antigen, the promoter activity of the late control region is induced by ≈1,000-fold above the basal level. By deletion and point mutagenesis, we define two domains of the late control region required for efficient induction with T antigen. Domain I is the minimal replication origin containing T-antigen binding site II. Domain II consists of the 72-base-pair (bp) repeat and a 19-bp downstream sequence up to nucleotide 270. Domains I and II should act synergistically because the absence of either one or the other decreases induction efficiency by 2 orders of magnitude. Though a complete copy of domain II is optimal, the origin-proximal 22-bp portion of this domain is sufficient. The 21-bp repeat, located between domains I and II, is dispensable for this induction, as are sequences located downstream from nucleotide 270 in the late orientation.**

A 400-base-pair (bp) noncoding region of simian virus 40 (SV40) DNA located between its early and late genes contains the early and late transcriptional promoter elements and the viral replication origin (1). This 400-bp origin region contains three palindromes, a 17-bp A+T-rich block, three copies of a 21-bp repeat, and two copies of a 72-bp repeat. SV40 early promoter resembles typical eukaryotic RNA polymerase II promoters in its structure and contains a "TATA" box located within the 17-bp A+T-rich block and six copies each of two overlapping C-rich oligonucleotides located within the 21-bp repeat (2–4). The 72-bp repeat contains an element that enhances transcription off of the SV40 early promoter (5, 6) and the promoters of several heterologous genes as well (7, 8).

The early viral protein large tumor antigen (referred to as T antigen hereafter) is the only known viral regulatory protein. T antigen is known to bind to the SV40 origin region at three sites (9–11), which is believed to mediate the initiation of replication and the autoregulation of early gene transcription brought about by T antigen (12–15). Binding site II contained within the minimal replication origin is obligatory for replication, while the flanking sites I and III enhance replication efficiency (14, 16). SV40 late gene expression, dormant at the early phase of infection, increases by about 1000-fold after the onset of replication. T antigen is believed to have a positive role in late gene expression (17), but it has not been resolved whether T antigen acts directly to stimulate late transcription or indirectly via its activation of replication (reviewed in ref. 18).

The sequences contained in the late promoter region are essentially those present in the early promoter region but in the reverse order and orientation. Late mRNA initiation sites are heterogeneous and are not preceded by a TATA box or any other component of classical eukaryotic RNA polymerase II promoters, indicating that the SV40 late promoter is structurally unique. Sequences of the late control region essential for promoter activity in the absence of T antigen and autonomous replication of the template were mapped recently. In an *in vivo* study using a long-term transformation assay (19) and in two *in vitro* transcription studies using HeLa cell extracts (20, 21), the 21-bp repeat was found to be a major component of the late promoter. In addition, in the *in vivo* study (19), the 72-bp repeat was found to provide a 4- to 5-fold enhancement.

In the present study, we examined the regulation of gene expression under the control of the SV40 late promoter, using a transient expression vector system *in vivo* under physiological conditions resembling those existing at the late phase of infection of permissive cells by SV40—namely, in the presence of T antigen and autonomous replication of the template.

MATERIALS AND METHODS

SV40 late-control-region fragments, having specific deletions (or a point mutation), were cloned upstream from the promoterless gene for chloramphenicol acetyltransferase (CAT) in the pSV0-cat vector as shown in Fig. 1. CV-1 and COS-1 monkey cells of low passage (22, 23) were transfected with 10 μg of form I CAT gene-containing plasmids (CAT plasmids) per dish by the calcium phosphate coprecipitation procedure (22), except that the coprecipitates were left on the cells overnight prior to glycerol-shocking. At 24 or 48 hr after glycerol-shocking, the cells were harvested and crude extracts were made by sonication (22).

The CAT activities of the extracts were assayed by measuring the conversion of chloramphenicol to its acetyl derivatives by fractionation involving TLC (22) or HPLC (unpublished data) using silica gel plates or columns with chloroform/methanol, 95:5 (vol/vol), as the solvent. The HPLC assay has the advantage of using inexpensive unlabeled chloramphenicol as the substrate for the CAT reaction and is rapid, automatic, accurate, and quantitative. Extracts were diluted, and lower levels were used in CAT assays if the percentage of acetylation exceeded 30%, indicating substrate

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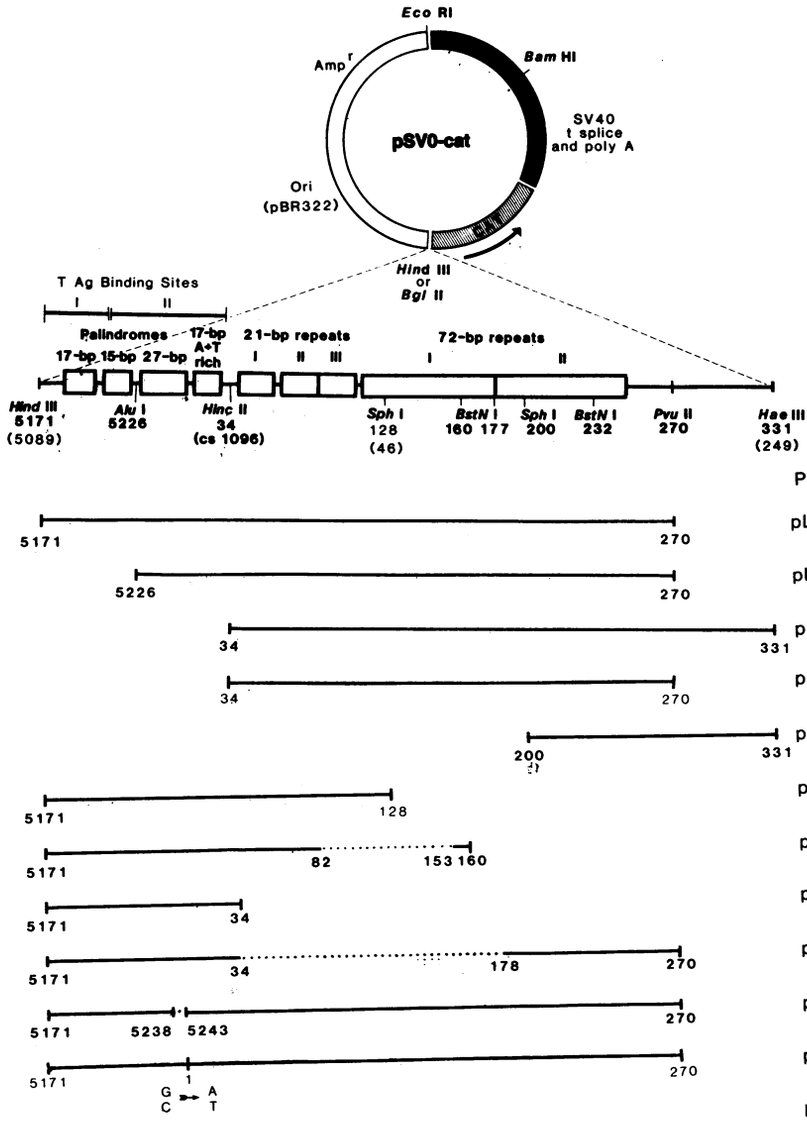
Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; bp, base pair(s); CAT, chloramphenicol acetyltransferase; CAT plasmids, CAT gene-containing plasmids.



PLASMID	UNITS OF CAT ACTIVITY: CV-1 48hr	CV-1 72hr	COS-1 48hr	COS-1 72hr
pLP1-cat	<0.01	<0.01	8.60	29.10
pLP2-cat	<0.01	<0.01	0.01	0.02
pLP3A-cat	0.02	0.04	0.07	0.24
pLP3B-cat	0.02	0.04	0.10	0.42
pLP5-cat	<0.01	<0.01	0.01	0.10
pLP13-cat	<0.01	<0.01	0.30	5.97
pLP7-cat	<0.01	<0.01	0.01	0.12
pLP8-cat	0.02	0.04	0.04	0.29
pLP9-cat	0.03	0.03	5.10	10.40
pLP11-cat	0.02	0.03	0.12	0.55
pLP12-cat	<0.01	<0.01	<0.01	<0.01
pEP1-cat	0.20	0.40	0.40	0.70

FIG. 1. Construction and analysis of chimeric SV40 late promoter (LP)- and CAT gene-containing plasmids. The open SV40 nucleotide numbers are according to the SV system (18), and those in parentheses are according to the Weissman system (1). T Ag, SV40 T antigen. SV40 LP region fragments LP1 through LP13 (shown as part of the corresponding CAT plasmids designated pLP1-cat, etc.) were obtained by restriction cleavage of wild-type or mutant SV40 DNAs as described earlier (19). Fragment LP12, with the point mutation indicated, was made from mutant *sp*1030 (ref. 13). Solid horizontal lines show the SV40 sequences present in the fragments, and the dotted lines denote internal deletions. Each fragment was cloned as a single copy at the unique *Hin*dIII or *Bgl* II cloning site of the pSV0-cat vector in the late orientation with respect to the CAT gene as determined by restriction mapping and DNA sequencing (19). Plasmid pEP1-cat contains the same SV40 origin region sequence as pLP1-cat, but in the opposite (early) orientation. The CAT activities of extracts of CV-1 or COS-1 cells transfected with the CAT plasmids, measured at 48 or 72 hr after transfection, are shown at the right as units per 5×10^5 cells (see *Materials and Methods* for the definition of unit).

exhaustion. When this precaution was followed, the plot of percent acetylation against reaction time was linear. Values of percent acetylation within the linear range were converted to CAT activities in units, with one unit being defined as the amount of enzyme required to acetylate 1 nmol of chloramphenicol per min at 37°C.

RESULTS

SV40 Origin Region Functions as an Efficient Promoter in the Early but Not the Late Orientation in the Absence of T Antigen. Plasmids pEP1-cat and pLP1-cat have the same 340-bp complete SV40 origin-region fragment cloned in the early or the late orientation, respectively, upstream from the CAT gene. In CV-1 monkey cells lacking T antigen, at 48 hr after transfection, pEP1-cat was expressed quite well, while pLP1-cat was not, even at extended periods of up to 72 hr after transfection (Figs. 1 and 2).

Late Promoter Activity Is Induced to High Levels in the Presence of T Antigen. Plasmid pLP1-cat, which was expressed poorly in CV-1 cells, was expressed with high efficiency in COS-1 cells (Fig. 2), which produce the SV40 T antigen constitutively (23). Accurate measurement of CAT activities in diluted extracts indicated an ≈1,000-fold induction of CAT activity in COS-1 cells compared to that in CV-1 cells in transfections involving pLP1-cat, whereas pEP1-cat showed a ≤2-fold difference under these conditions (Fig. 1).

The expression of pLP1-cat also could be induced in CV-1 cells themselves with T antigen produced by infection of the transfected cells with SV40 (Fig. 3), though the induced

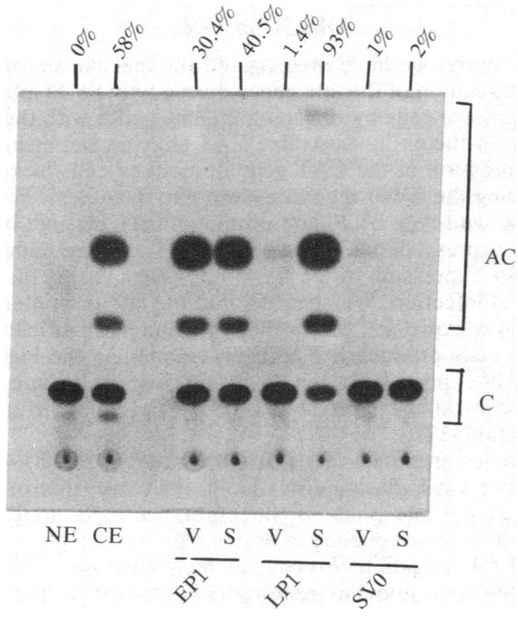


FIG. 2. The promoter efficiencies of the SV40 origin region in the early (EP1) or late (LP1) orientation measured in CV-1 (denoted as V) and COS-1 (denoted as S) cells by the TLC assay for CAT activity (22). SV0 refers to the promoterless CAT gene-containing vector pSV0-cat; NE and CE are reaction controls and refer, respectively, to the negative control lacking CAT enzyme and the positive control having 0.4 units of commercial CAT (P-L Biochemicals). The percentage conversion of chloramphenicol (C) to its mono- and diacetylated products (AC) in each reaction is indicated at the top.

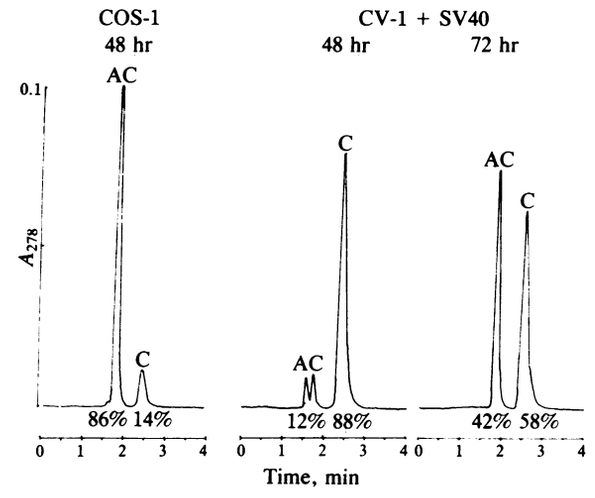


FIG. 3. The expression of the wild-type SV40 late promoter- and CAT gene-containing plasmid pLP1-cat in COS-1 cells (*Left*) and in CV-1 cells infected with SV40 (*Right*), measured at 48 or 72 hr after transfection by the HPLC assay for CAT activity. Tracings of the chromatographs are shown. Cells, transfected with the plasmids overnight, were glycerol-shocked (22). At 6 hr after the shocking, some plates of the transfected CV-1 cells were infected with SV40 at a multiplicity of infection of 20 plaque-forming units per cell. Peaks corresponding to chloramphenicol (C) and its mono- and diacetyl derivatives (AC), appearing with retention times of 2.55 and 1.85 min, respectively, are indicated, and peak area percentages are shown below. Extracts of CV-1 cells transfected with pLP1-cat but not infected with SV40 exhibited <2% acetylation at 48 or 72 hr after transfection (not shown).

level was an order of magnitude lower than that in COS-1 cells. A possible reason for this decrease could be the competition by the autonomously replicating SV40 DNA with the CAT plasmid (which is also replication-capable) for the available T antigen. For this reason, and for reasons of convenience, COS-1 cells were used as the source of T antigen in subsequent experiments.

The Minimal Replication Origin Is Essential for Maximum Induction of Late Gene Expression. The effect of various deletions in the late control region on CAT gene expression in CV-1 and COS-1 cells is tabulated in Fig. 1. The basal, uninduced levels of expression in CV-1 cells of a majority of these CAT plasmids was low, except those of a few lacking the minimal replication origin or the 21-bp repeat for reasons that are not clear at present.

Late promoter segments designated LP2, LP3A, LP3B, LP5, LP11, and LP12 contain lesions within the minimal replication origin (13, 14). CAT plasmids having these segments were replication-negative (data not shown) and, as a rule, were induced with efficiencies that were 2–3 orders of magnitude lower than that of the replication-competent pLP1-cat in COS-1 cells (Fig. 1), underscoring the importance of replication towards late gene expression. Significant differences were noted in the induction efficiencies in COS-1 cells of members of this group, shedding some light on induction achieved in the absence of replication. Plasmid pLP12-cat, having a deleterious point mutation in T-antigen binding site II (13), was uninducible. On the other hand, plasmid pLP11-cat, having a 4-bp deletion in site II (23), was moderately inducible (Fig. 1). These results point out the essentiality of site II for the induction of late gene expression, but it remains to be seen if, as reported by other workers (24, 25) using different strategies and mutants, there is a correlation between the efficiency of T-antigen binding and the extent of induction.

Plasmids pLP3A-cat and pLP3B-cat, lacking T-antigen binding sites I and II entirely, were induced moderately (Fig. 1). Both of these constructs contain the weak binding site III, which might play a minor role in the induction in the absence of sites I and II. All of the three T-antigen binding sites, as well as the origin-proximal portion of the 72-bp repeat required for induction (see below), are absent in pLP5-cat, explaining its low induction efficiency (Fig. 1).

The 72-bp Repeat Is Essential but the 21-bp Repeat Is Dispensable for Induction of Late Gene Expression. The CAT plasmids having the late control region segments LP7, LP8, LP9, and LP13, contain the intact minimal origin (14, 16) and are all capable of replication in COS-1 cells as verified in this study (data not shown). However, autonomous replication, though necessary for maximum induction of late promoter activity, was not sufficient in itself, as shown by the low induction efficiencies of pLP7-cat (having the minimal origin and the 21-bp repeat) and pLP8-cat (having only the minimal origin), both of which lack the 72-bp repeat (Fig. 1). On the other hand, plasmid pLP9-cat, lacking the 21-bp repeat entirely but retaining one copy of the 72-bp repeat and a 19-bp sequence located immediately downstream from it up to nucleotide 270, was induced to high levels in COS-1 cells, approaching the induction efficiency of pLP1-cat (Fig. 1). These results show that the 72-bp repeat is essential but the 21-bp repeat is dispensable for the activation of the late control region.

When the minimal origin is present, though a complete copy of the 72-bp repeat is optimal, the origin-proximal 22-bp portion of the 72-bp repeat (contained in pLP13-cat) was sufficient for induction (Fig. 4). Plasmids pLP1-cat and pLP9-cat showed a lag period of about 24 hr after transfection (or 6 hr after glycerol-shocking), after which the induction was nearly linear, proceeding at high rates. Plasmid pLP13-cat exhibited a greater lag period, after which it was induced at a high rate equaling that of pLP9-cat. Two other plasmids,

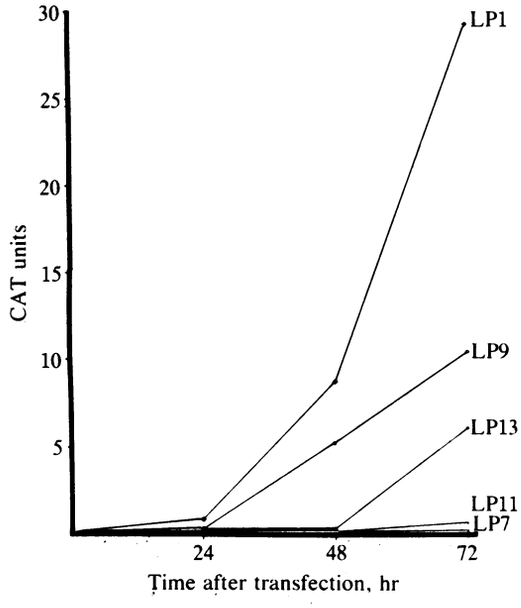


FIG. 4. Time course of CAT gene expression in COS-1 cells transfected with plasmids containing the CAT gene and SV40 late promoter segments LP1, LP9, LP13, LP11, and LP7. CAT activities of extracts of the transfected cells were determined at 24, 48, and 72 hr after transfection and are shown as units of CAT activity per 5×10^5 cells (see *Materials and Methods* for unit definition).

the replication-competent pLP7-cat and the replication-negative pLP11-cat, exhibited induction efficiencies much lower than that of pLP13-cat.

Based on all of these results, we define two distinct but noncontiguous domains of the SV40 late control region required simultaneously for induction with T antigen (Fig. 5). Domain I corresponds to the minimal replication origin including T-antigen binding site II. Domain II includes the 72-bp repeat of which the origin-proximal 22-bp portion is sufficient. The 21-bp repeat located between these two domains is dispensable for the induction, as are sequences located downstream from nucleotide 270 in the late orientation.

DISCUSSION

In this study, we have investigated the mechanism of temporal regulation of the late genes during lytic SV40 infection of permissive cells by replacing the late genes with the gene coding for the easily assayable CAT enzyme and measuring the expression of the CAT gene in monkey cells lacking or producing the SV40 regulatory protein, T antigen. We find that the wild-type SV40 late promoter CAT plasmid is very poorly expressed in CV-1 cells lacking T antigen, mimicking the poor expression of the SV40 late genes during the early phase of infection. We also find that the late-promoter CAT plasmid is expressed with a 1000-fold increased efficiency in COS-1 cells producing T antigen, resembling the high efficiency of expression of the SV40 late genes at the late phase of infection after the production of T antigen and the onset of replication (18).

Having correlated the functional state of the SV40 late promoter CAT plasmids in COS-1 cells with that of SV40 DNA at the late phase of infection, we have gone on to determine the sequence requirements at the late control region for induction to occur at high efficiency. We have identified two domains required simultaneously for induction, the first being the minimal replication origin and the second being the 72-bp repeat. These two domains act in a synergistic or cooperative fashion, since either domain alone does not give rise to >10-fold induction, but, together, they give rise to an induction efficiency that is greater by 2 orders of magnitude (Fig. 1).

The late promoter region can be activated in the absence of autonomous replication, as shown by the inducibility of the replication-negative plasmids pLP11-cat, pLP3A-cat, and pLP3B-cat (Fig. 1). However, the induction efficiency in these cases does not exceed 20-fold at 72 hr after transfection, in contrast with induction efficiencies of 350-fold or more obtained with the replication-competent plasmids pLP1-cat, pLP9-cat, and pLP13-cat (Fig. 1). Thus, autonomous replication of the template is essential (but not sufficient) for maximum activation of the late control region.

The 72-bp repeated sequence constitutes the second domain necessary for induction of late-promoter activity. As mentioned in the *Results* (provided the minimal origin is also present), the origin-proximal 22-bp portion of the 72-bp re-

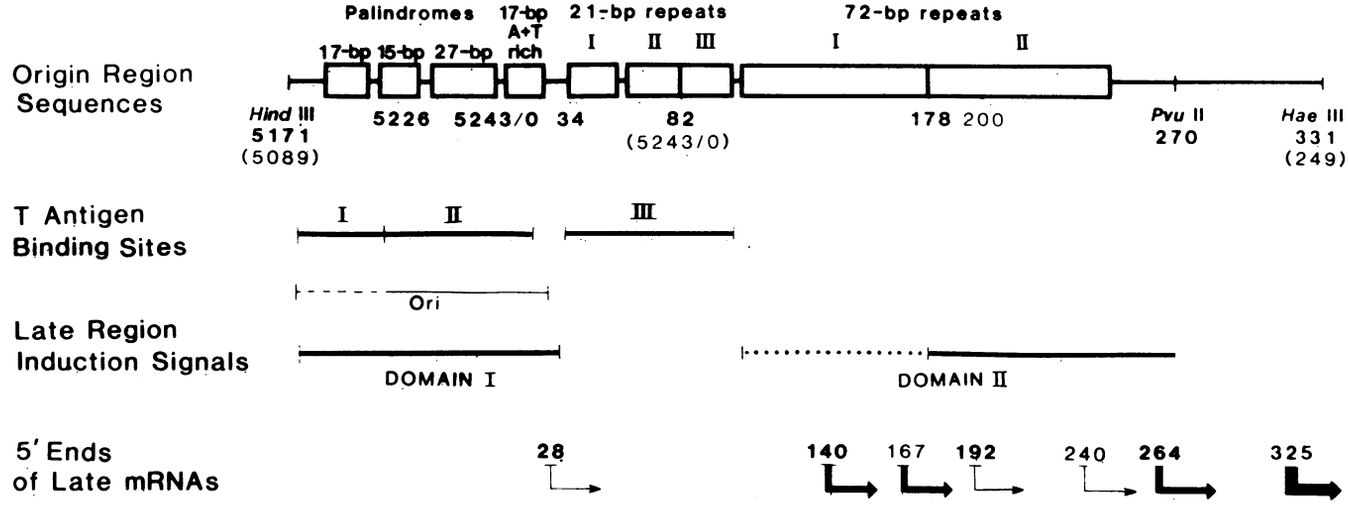


FIG. 5. Sequences present in domains I and II of the SV40 late upstream control region, identified in this study as simultaneous requirements for maximum late gene induction in permissive cells producing T antigen. The SV40 numbering is explained in Fig. 1. The boundaries of the T-antigen binding sites (9–11), the minimal replication origin denoted as "ori" (14, 16), and the initiation sites of SV40 late mRNAs (26, 27) are also shown. Domain II shown includes the 72-bp repeat and the immediate downstream sequence up to nucleotide 270. The dotted line in domain II indicates sequences reiterated in it. Though a complete copy of domain II is optimal for induction, the origin-proximal 22-bp of this domain is sufficient (provided domain I is also present).

peat is sufficient for induction, whereas the origin-distal portion of the 72-bp repeat [containing the enhancer core sequence and flanking Z DNA-forming elements, shown previously to be essential for the T antigen- and replication-independent enhancement of transcription off of the SV40 early promoter and heterologous promoters by the 72-bp repeat (2, 5–8, 28, 29)] is dispensable.

The SV40 late mRNA 5′ ends are heterogeneous and map at nearly the same locations in the genome, with the mapping studies done under diverse conditions such as in permissive cells *in vivo* at the late phase of infection [i.e., upon induction (26, 27)], in *Xenopus* oocytes *in vivo* without induction (27), and by *in vitro* transcription (20, 21). A number of these 5′ ends are located within the 72-bp repeat region, which might account at least in part for its function in the induction of late gene expression.

As referred to in the *Results*, T antigen is likely to mediate the participation of the minimal replication origin on the induction of late gene expression, probably through its autoregulation and replication initiation functions. The 72-bp repeat is not required for replication (16) and does not contain any T-antigen binding sites as revealed by *in vitro* binding studies (9–11). Therefore, a cellular factor is a possibility to mediate the effect of the 72-bp repeat by binding to this sequence and inducing the late gene expression.

Three recent studies have indicated that the 21-bp repeat is a major component of the SV40 late promoter. One of these, an *in vivo* study (19), employed a stable transformation assay with the templates integrated into the cell DNA and was carried out in the absence of T antigen. The other two were *in vitro* transcription assays, using HeLa cells (20, 21), in which the activity of the late promoter measured is likely to be its basal, uninduced level. In contrast with these studies, our present study has been performed *in vivo* under physiological conditions resembling those prevailing at the late phase of infection of permissive cells by SV40—i.e., in the presence of T antigen and with the templates existing in the plasmid state. Under these conditions of induction, the 21-bp repeat is dispensable for late promoter activity. Also, in disagreement with the recent results of Ernoult-Lange *et al.* (30), we find that sequences located downstream from nucleotide 270 in the late orientation are dispensable for induction (compare pLP3A-cat and pLP3B-cat in Fig. 1), confirming earlier results with viable deletion mutants of SV40 lacking these sequences (31).

Overall, the SV40 late upstream control region (and possibly those of other papovaviruses) is a unique one. It does not contain any classical eukaryotic RNA polymerase II promoter sequences. It consists of a medley of sequences including the minimal replication origin (containing T-antigen binding sites that also mediate the repression of the early gene expression), a signal(s) for induction of late gene expression located within the 72-bp repeat, and a series of mRNA initiation sites located within and immediately downstream from the 72-bp repeat (Fig. 5). After the production of T antigen, all these sequences are activated in unison, resulting in the high level of expression of the late genes of SV40.

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